

Genetic Improvement through Selection of Different *Stevia rebaudiana* Genotypes

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ABSTRACT

The present investigation was carried out at Faculty of Agriculture Saba Basha, Alexandria University and Sabahia Agricultural Research Station during two successive seasons 2013-2014 and 2014-2015 from nature population of *Stevia* (*Stevia rebaudiana* Bertoni) in order to study the genetic improvement through selection for different *Stevia* genotypes. Nineteen stevia accessions were collected from healthy plants of nature population and selected randomly. The data showed high significant variations between all tested genotypes and there is high genetic diversity between these genotypes and this might be, due to the open pollination for these genotypes for several years and these genotypes could be used in the future in breeding programme. Also, it can be concluded that the difference in rebaudioside A due to the difference in the genetic and the negative correlation between stevioside and rebaudioside-A. can be partially explained by the biosynthetic relationships between the individual glycosides because stevioside is the substrate for the synthesis of rebaudioside A.

Key words: *Stevia rebaudiana*, selection, HPLC, RAPD-PCR

INTRODUCTION

Stevia rebaudiana Bertoni is a genus of about 200 species of herbs and shrubs in the sunflower family (Asteraceae), the most valuable tropical medicinal plant. It's a natural sweet herb native of northeastern Paraguay and today it is cultivated around the world (Savita *et al.*, 2004). *Stevia* is a diploid plant, having 11 pairs of chromosomes (Frederico *et al.*, 1996) with a critical day length of 13 hours (Zaidan *et al.*, 1980). *Stevia rebaudiana* Bertoni grows up to 1 m tall (Mishra *et al.*, 2010). The plant is a perennial herb with an extensive root system and brittle stems producing small, elliptic leaves. Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants comes from its chemical substances that produce a definite physiological action on the human body. Alkaloids, tannins and polyphenols are important bioactive constituents of *Stevia* (Edeoga *et al.*, 2005).

S. rebaudiana (commonly referred to as honey leaf, candy leaf and sweet leaf) is rich in terpenes and flavonoids. The basic raw material for the production of stevioside is the leaves of the stevia plant. The leaves of stevia naturally contain a mixture of 8 sweet diterpene glycosides: C, D stevioside, steviolbioside, rebaudiosides (A, B, and E) and dulcoside A (Geuns, 2003). Stevioside, one of the *Stevia* glycosides, is about 300 times sweeter than saccharose and is present with an average of 4-20% in the dry matter of the plant leaves, which primarily depends on cultivar characteristics of plants and basic agricultural techniques (Geuns 2000) and can be particularly beneficial to those suffering from obesity, diabetes mellitus, heart disease and dental caries (Ghanta *et al.*, 2007). Dry leaves of stevia are sweeter approximately 10 to 15 times than sucrose (Raymond, 2009) while glycemic index is zero, so it is a sweetener with no caloric value (Kroyer, 2010; Seema, 2010; Puri *et al.*, 2011) and with proven non-toxic effect on human health (Barriocanal *et al.*, 2008).

The leaves of stevia are used as a food additive and a sweetener in food products, a green powder obtained by grinding of dried green leaves (Mishra *et al.*, 2010), a white powder obtained by depigmentation process of green powder (Brandle *et al.*, 1992) and a solution obtained by different extraction methods of stevioside and rebaudioside A from green powder (Abou-Arab *et al.*, 2010). Stevia leaf extract shows a high level of antioxidant activity, as well as, the variety of phytochemicals such as phenolic compounds, which is directly associated with the removal of free electrons and superoxide radicals (Thomas and Glade, 2010; Benzie and Watchel-Galor, 2011). Therefore, stevia plant has significant potential for use as a natural antioxidant (Shukla *et al.*, 2009; Ahmad *et al.*, 2010; Kim *et al.*, 2011). In recent years, stevia products find widespread use in the food industry (Savita *et al.*, 2004; Midmore and Rank, 2006). Steviol glycosides are used as a sweetener in many industrial foods, such as soft drinks or fruit juices (Goyal *et al.*, 2010), desserts, sauces, delicacies, sweet corn, bread, biscuits and a

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table sweetener. Stevia diterpene replace sucrose in: cereals (muesli), pickles, yoghurt (Amzad-Hossain *et al.*, 2010), candy (Goyal *et al.*, 2010), soybeans and soy sauce (Amzad-Hossain *et al.*, 2010) and seafood (Goyal *et al.*, 2010).

Randomly amplified polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR) based on procedure of (Williams *et al.* 1990). RAPD markers are usually dominant, with polymorphisms between individual plants indicated by the presence or absence of a particular rapd fragment. RAPDs have widely been used for map construction and linkage analysis because of their simplicity and relatively low cost (Reiter *et al.* 1992). As part of laying the foundation for molecular breeding, RAPD markers used to study the genetic polymorphic aiming to construct a genetic map of the stevia genome (Banerjee *et al.*, 1999). Thiyagarajan and Venkatachalam (2015) reported that the genomic DNA polymorphism and phytochemical variation of *Stevia rebaudiana* Bertoni was investigated by RAPD-PCR for initial PCR screening, about hundred oligo-nucleotide primers were used to amplify the genomic DNA from three accessions of *S. rebaudiana* (L1 to L3).

HPLC used to analyze stevioside extracted from the leaf and callus of *Stevia rebaudiana* Mahmud *et al.* (2014). The stevioside in each sample were analyzed by comparing their retention times with those of the standards. They maintained that the retention time (RT) of stevioside for leaves was found 14.96 and for callus 13.81 mins. The percentage of stevioside content from leaves and callus was 12.19% and 12.62% respectively. This investigation was carried out to study the following aspects, morphological characteristics such as plant height (cm), number of secondary branches per plant, fresh weight of leaves (gm/plant), dry weight of leaves (gm/plant), fresh weight of stems (gm/plant) and dry weight of stems (gm/plant), on *Stevia* plants, calculate the content of Stevioside and Rebaudioside A and identify the genetic polymorphism in *stevia rebaudiana* population via RAPD-PCR.

MATERIALS AND METHODS

The present investigation was carried out at Faculty of Agriculture Saba Basha, Alexandria University and Sabahia Agricultural Research Station during two successive seasons 2013-2014 and 2014-2015 upon nature population of *Stevia* (*Stevia rebaudiana* Bertoni). Nineteen stevia accessions (*Stevia rebaudiana* Bertoni) were collected from healthy plants of nature population and selected randomly, plants were multiplied vegetatively through micro-cuttings in of 10:15 cm with 3-4 leaves. The micro-cutting propagation was carried out by cutting the young apical shoots (YAS) of matured stevia accessions towards the apex. Five plants

per an accession were made from each genotype. The excised cuttings were dipped into Indole-3-butyric acid (IBA) for 15 minutes in order to allow the auxin get absorbed into the cut stem through the xylem, and in order to stimulate the cuttings to sprout roots in a mist chamber. Upon rooting the young plants were transferred to a nursery section in small polyethylene bags under a shade and were made to remain there for two weeks. This stage was necessary to harden the young growing plants. The soil type used in this experiment was a mixture of sand and loam; all the plants were subjected to the same conditions of growth and made to develop under the same climatic condition in Sabahia Agricultural Research Station. After two weeks young plants were transferred to a larger polyethylene bags and finally taken to field. When the plants became three months old they were evaluated on the field and subjected to various analyses.

Morphological variations of *Stevia* genotypes:

Plant height (cm) was expressed in cm, as the ratio between the measurements from the soil surface to the highest point of the plant, obtained from five plants of each plot; number of secondary branches, calculated as the mean number of secondary branches per plant (BP); fresh weight of leaves (g) calculated as yield of fresh matter (FM) in g /plant; dry weight of leaves (g) calculated as yield of dry matter (DM) in g/plant; fresh weight of stems (g) calculated as yield of fresh matter (FM) and expressed in g/plant weighing five plants per plot; dry weight of stems (g): yield of dry matter (DM) was expressed in g/plant; moisture contents (%): calculated as (fresh weight- dry weight)/Fresh weight*100

Extraction and estimation of *Stevia* sweeteners:

Stevia leaves were dried in an electric oven (E. Schulz & Co. Inh. Franz. Skorzewsh KG at 50°C). *Stevia* sweeteners were obtained from *Stevia* International Company for Agra industrial Projects (SKAP). Stevioside standard preparation was carried out according to Nishiyama *et al.* (1992) as follows: dried Leaves (10 g leaves of *Stevia rebaudiana* Bertoni obtained from Sigma) were extracted by soaking leaves in 1.0 liter of water nearly boiling (85°C) for 30 minutes. The resulting liquid fraction was separated by buchner filtration and the residue was washed with an additional volume of hot water (50 ml). The aqueous solution was concentrated by lyophilization (Edwards Model EF03, England) to be 50 ml and defatted by ethyl acetate then extraction with isobutyl alcohol (150 ml). The aqueous phase was discarded and the organic solution was evaporated by rotary evaporator (Type 349, James Jobling and Co. Ltd. England) at 70°C until drying was obtained. The dried extract was dissolved in

hot methanol (100 ml) and kept over night to crystallize. The crystals were separated by filtration and re-dissolved again in boiling methanol (50 ml). This solution was clarified with active charcoal (B.D.H. Laboratory Chemicals Division. The procedure was repeated three times until the formation of colorless crystals was observed. The pure solution of the stevioside prepared (pH of 9) was subjected to HPLC. At the same time an authentic pure stevioside was obtained from Nu Naturals Inc. U.S.A.

Extraction of Stevia sweeteners from leaves was carried out by 0.5 gm of dry stevia leaves was ground and dissolved in 0.5 ml methanol and put in shaking and heating for 30 minutes at 70° C then kept in room temperature for cooling then abukhner funnel was used for filtration using A filter paper one time after that we used Activated charcoal for filtration another time finally it were kept the filtrate frozen until analysis. Stevia (*Stevia rebaudiana* Bertoni) leaves extract was separated and identified on HPLC as follows: Stevioside and other sweet components standard as prepared above were filtered through a millipore membrane (13 mm. diameter, 0.5 µm pore size) were subjected for determination by chromatography with stevioside standard as internal standard. Different extracts of Stevia leaves were injected for chromatography Acetonitrile with HPLC grade (Fisons Co. England) was used in this study as mobile phase. HPLC separation was carried out on 210 nm (Agilent 1200PDA detector); Eclipse plus C18 column (3.5µm 4.6x250 mm); linear gradient over 20min (84:55% CH3CN in H2O/ 0.1% TFA); flow rate 2.0 mL/min. Injection volume: 70 µl at ambient temperature (25°C). All the conditions used were according to Makapugay *et al.*, (1984). For each samples identification quantification and the retention time were as described by Makapugay *et al.*, (1984). Area under each peak was used to calculate the percent of each compound.

Random amplified polymorphic DNAs (RAPD) analysis of Stevia genotypes:

DNA is amplified by the polymerase chain reaction (PCR) using arbitrary short (10 nucleotides) primers (Williams *et al.*, 1990). RAPD has become an important technique for population genetic studies since the amplified products provide random representation of both coding and non-coding regions across the whole genome. DNA was extracted following (Murray and Thompson, 1980) with minor amendments as follow. 50 mg samples of of Leaves of Stevia (*Stevia rebaudiana* Bertoni) material was ground with pestle and motor in 1ml 2X CTAB (1M tris (ph8), 0.5M EDTA (ph8), 5M NaCl, 20gm CTAB (Cetyl-tetramethyl ammonium bromide) in 1000ml) containing 25µl mercaptoethanol, 10mg polyvinyl pyrrolidone (pvp). About 750 µl well ground paste was taken in to 1.5 ml labeled eppendorf tube. Tubes were inverted gently to mix the ingredients incubated at 65°C for 45 minutes in water bath and centrifuged 12,000 rpm for 10 minutes. Then in new eppendorf tube taken 700µl supernatant, 350µl 1M tris (ph 8), 350µl chloroform: isoamyle alcohol(24:1), 5µl proteinase K. Tubes were inverted gently to mix the ingredients and centrifuged 12,000 rpm 10 minutes. About 400 µl supernatant were taken carefully in to new labeled eppendorf tube, 400µl chloroform: isoamyle alcohol (24:1) and centrifuged 12,000 rpm 10 minutes. 400 µl supernatant were taken carefully in to new labeled eppendorf tube 700 µl chilled isopropanol was added to each tube and mixed gently. Tubes were incubated at 4 °C over night and centrifugation 12,000 rpm for 10 minutes. Supernatant was discarded and DNA pellet was rinsed with 70% ethanol. DNA pellet was air dried. The pellet was dissolved in 100 µl TE buffer (10 mM Tris, 1mM EDTA and PH: 8.0). RNAase-A was used to remove RNA from the DNA. For this purpose RNAase-A (0.20 µl of RNAase-A procured from Gene Link, USA) 40 µg was used and DNA samples were kept at 37°C for 30 minutes in water bath. DNA quantification was carried out in spectrophotometer. A 20 ng dilution of DNA was made in deionized water to use in Polymerase Chain Reaction (PCR). DNA samples were stored at -20 °C.

Table 1. Primers and their oligonucleotide sequences used

| Primer number | Primer Code | Sequence 3'---5' |
|---------------|-------------|--------------------|
| 1 | OPN-04 | 5'- GACCGACCCA -3' |
| 2 | OPD-05 | 5'- TGAGCGGACA -3' |
| 3 | OPC-05 | 5'-GATGACCGCC -3' |
| 4 | OPB-07 | 5'-GAAACGGGTG -3' |
| 5 | OPN-10 | 5'-ACAACGGGG -3' |
| 6 | OPG-12 | 5'-CAGCTCACGA -3' |
| 7 | OPQ-12 | 5'- AGTAGGGCAC -3' |
| 8 | OPQ-14 | 5'-GGACGCTTCA -3 |

The polymerase chain reaction (PCR) conditions:

RAPD analysis was carried out using eight oligonucleotide primers (Table 1) that were selected from the Operon Kit (Operon Technologies Inc., Alabameda, CA). The polymerase chain reaction mixture (25 μ l) consisted of 13 μ l master mix (Promega) *Taq* DNA polymerase; 2 μ l of genomic DNA, 2 μ l primer, 8 μ l deionized water. PCR amplification was performed in a Biometra T1 gradient thermal cycler for 35 cycles after initial denaturation for 5min at 94°C. Each cycle consisted of denaturation at 94°C for 1min; annealing at 36°C for 1min; extension at 72°C for 2min and final extension at 72°C for 5min (Williames, *et al.* 1990). Amplification products were separated on 2% Agarose gels at 100 volts for 1.30 hrs with 1 x TBE buffer. To detect ethidium bromide/DNA complex, Agarose gels were examined on ultra violet transilluminator (302nm wavelength) and photographed. Using 100pb Plus DNA ladder, ready-to-use (Gene Ruler, Fermentas, and Life Sciences), the lengths of the different DNA fragments were determined. For each sample, the reproducible DNA bands from two runs were scored for their presence or absence.

Fragments scored as present/absent. Fragment scoring and lane matching performed automatically on digital images of the gels, using Phoretix 1D advanced Version 4.00 (Phoretix International, Newcastle upon Tyne, UK). All but the faintest bands scored, where necessary scores and matches corrected manually. Clustering methods and similarity coefficients were tested using the procedures SIMQUAL, SAHN, and TREE from the program NTSYSpcversion 2.10 (Applied Biostatistics, Setauket, New York, USA). The clustering methods UPGMA, WPGMA, Complete-link, and Single-link were applied in all possible combinations with the similarity coefficients Dice, Jaccard and simple matching. Phoretix electrophoresis gel image analysis, ID software was used for scanogram tracing of protein bands of the tissues. The molecular weights of protein bands were determined against protein calibration kits (Spectra™ Multicolor Broa Range Protein Ladder; fermentas life sciences under the license for Strep-tag® technology). Data matrices were entered into the NTSYS (Numerical Taxonomic and Multivariate Analysis System) program, version 2.1, Applied Biostatistics Inc. (Rohlf, 2000). Similarity coefficients were used to construct dendrograms using the UPGMA (Unweighted Pair Group Method with Arithmetic average) and the SAHN (Sequential Agglomerative Hierarchical Nested clustering) routing in the NTSYS software. One Way ANOVA in

completely randomized design was used to reveal the significant differences among the samples. The L.S.D. (least significant differences) test was conducted to identify the significant differences among the means at 5% level of probability. Comparison of the mean values is usually calculated after an ANOVA.

RESULTS AND DISCUSSION

Morphological characteristics:

Results in Table 2 indicated high significant variations among all studied *stevia* genotype in relation to plant height (cm). The highest plant height values were recorded to genotype-7 by mean 123 cm followed by genotype 17 in average (113.87 cm) and genotype 5 in average (112.25 cm), while genotypes 13, 15 and 10 were the shortest genotypes in average 48, 43.75 and 41.25 cm, respectively. Analysis of variance in Table 2 showed high significant variation between all the *stevia* genotypes in relation to plant height by L.S.D._{0.05}=12.64, and also data indicated that no significant variations were observed among genotypes 2 and 3. Huge number of secondary branches was observed to the genotype 3 by mean 4.5 branch/plant, followed by genotypes 7, 11 and 13 showed an average 3.5 branch/plant and were no significant variation between them with L.S.D.=2.04. On the other hand no significant variation was observed between the other genotypes in relation to current traits (Table 2).

Data in Table 2 showed that genotype 14 has the highest value in fresh weight of leaves (g) by an average of (9.66 g) followed genotype 19 (6.19 g), genotype 9 (5.98 g) and genotype 16 by 5.95 g with L. S. D_{0.05} = 2.86. The results showed high significant variation between all genotypes of *stevia* in fresh weight of leaves (g). The lowest value was recorded to genotype 2 by average (2.45 g) as showed in Table 3 and Figure 4. Data in Table 2 showed that genotype 19 has the highest value in dry weight of leaves (g) by an average of (2.34 g) followed genotype 14 (1.84 g) and genotype 16 (1.63 g) with L.S.D_{0.05}=1.57. The results showed that all genotypes aren't significantly differed from each other except genotype 2 who recorded the lowest dry weight of leaves (0.5 g) as showed in Table 3 and Figure 5. Finally, data in Table 2 showed that genotype 18 has the highest value in moisture content/leaves (%) by an average of (84.83%) followed genotype 17 (83.90%) and genotype 9 (82.78%) by L.S.D_{0.05}=2.23. The results showed high significant variation between all genotypes of *stevia* in moisture content/leaves (%) and the lowest value was recorded to genotype 19 by average (62.26 %) as showed in Table 2.

Table 2. Plant height(cm) and Number of branches/ plant, Fresh, Dry weight/leaves (gm) and Moisture content/leaves (%) of nineteen genotypes of *Stevia rebaudiana* Bertoni from stem cuttings during 2014-2015 season

| Character | Plant height (cm) | Number of secondary branches/ plant | Fresh weight/leaves/ Plant (gm) | Dry weight /leaves/ plant (gm) | Moisture content / leaves (%) |
|-------------|------------------------|-------------------------------------|---------------------------------|--------------------------------|-------------------------------|
| Genotype 1 | 77.75 ^{fg} | 2.0 ^b | 3.17 ^b | 0.59 ^a | 81.08 ^b |
| Genotype 2 | 98.50 ^{bcde} | 2.5 ^{ab} | 2.45 ^b | 0.51 ^a | 78.76 ^b |
| Genotype 3 | 98.5 ^{bcde} | 4.5 ^a | 5.00 ^{ab} | 1.12 ^a | 77.08 ^{ab} |
| Genotype 4 | 83.75 ^{efg} | 3.0 ^{ab} | 3.83 ^b | 0.88 ^a | 76.98 ^b |
| Genotype 5 | 112.25 ^{ab} | 2.0 ^b | 5.23 ^{ab} | 1.25 ^a | 76.25 ^{ab} |
| Genotype 6 | 108.25 ^{abc} | 2.5 ^{ab} | 4.98 ^a | 1.35 ^a | 74.41 ^a |
| Genotype 7 | 123.00 ^a | 3.5 ^{ab} | 4.00 ^b | 0.76 ^a | 80.68 ^b |
| Genotype 8 | 84.50 ^{efg} | 2.0 ^{ab} | 3.80 ^b | 0.91 ^a | 76.60 ^b |
| Genotype 9 | 80.00 ^{efg} | 2.0 ^b | 5.98 ^{ab} | 0.80 ^a | 82.78 ^{ab} |
| Genotype 10 | 41.25 ^h | 2.6 ^{ab} | 3.15 ^b | 0.62 ^a | 79.09 ^b |
| Genotype 11 | 68.50 ^g | 3.5 ^{ab} | 3.76 ^b | 0.79 ^a | 77.85 ^b |
| Genotype 12 | 67.50 ^g | 2.5 ^{ab} | 4.99 ^{ab} | 1.14 ^a | 77.27 ^{ab} |
| Genotype 13 | 48.00 ^h | 3.5 | 4.33 ^b | 0.95 ^a | 77.80 ^b |
| Genotype 14 | 91.75 ^{cdet} | 2.0 ^b | 9.66 ^a | 1.84 ^a | 81.26 ^a |
| Genotype 15 | 43.75 ^h | 3.0 ^{ab} | 5.15 ^{ab} | 1.21 ^a | 75.64 ^{ab} |
| Genotype 16 | 66.00 ^g | 2.0 ^b | 5.95 ^{ab} | 1.63 ^a | 72.68 ^{ab} |
| Genotype 17 | 113.87 ^{ab} | 2.5 ^{ab} | 3.35 ^b | 0.55 ^a | 83.90 ^b |
| Genotype 18 | 50.00 ^h | 0.0 ^b | 5.79 ^{ab} | 0.86 ^a | 84.83 ^{ab} |
| Genotype 19 | 96.63 ^{bcdef} | 0.0 ^b | 6.19 ^a | 2.34 ^a | 62.26 ^a |
| LSD=0.05 | 12.64 | 2.04 | 2.86 | 1.57 | 2.23 |

Data in Table 3 showed that genotype 19 has the highest value in fresh weight of stems (g) by average 9.47 g followed genotype 16 (8.42 g) and genotype 4 (7.97 g) with L.S.D_{0.05}=5.43. The results showed that all genotypes were not significantly differed from each other except genotype 10 who recorded the lowest fresh weight of stems was genotype 10 by an average of (3.18 g) as showed in Table 3. Data in Table 3 showed that genotype 19 has the highest value in dry weight of stems (g) by average of (2.15) followed genotype 6 (1.74 g) and genotype 1 (1.6 g) with L.S.D_{0.05}=1.26. The results showed significant variations between all genotypes of stevia in dry weight of stems and detected the lowest value for genotype 10 by average (0.5 g) as shown in Table 4 and Figure 7. Results in Table 3 showed that genotype 3 has the highest value in moisture content/ stems (%) by average of (84.43%) followed genotype 11 (83.02%) and genotype 18 (82.85%) by L.S.D_{0.05} = 4.29. The results showed significant variation between all genotypes of stevia in moisture content of stems. The lowest value was recorded to genotype 1 by average (74 %) as showed in Table 3.

The previous data showed that high significant variations between all the genotypes in relation to the morphological characteristics had achieved. That means

there is high genetic diversity between these genotypes due to the open pollination for these genotypes several years and these genotypes could seed in the future breeding programme.

These results are in agreement that accepted by Abdullateef and Osman (2011) who's evaluated 10 stevia accessions in Malaysia using morphological parameters and collected data were subjected to 't' test analysis. Also, our results are agreed with Kassahun *et al.*, (2013) worked on some morphological character on *Stevia rebaudiana* Bertoni. Data on survival count, survival percentage, number of branches/seedlings, number of leaves/branches and number of leaves/seedlings were recorded. The results showed that Mean squares from analysis of variance revealed the existence of a very highly significant influence ($P < 0.001$) of cutting position, node number and interaction effect of cutting position with node numbers on all the parameters considered in the study. The current work is agreed with Shizhen, and Wanzhong (1988) studied the variation of 14 important quantity character of *Stevia rebaudiana* B. and the influence of different density on the variation.

Table 3. Fresh weight /stems (g) and Number of branches/ plant of nineteen genotypes of *Stevia rebaudiana* Bertoni from stem cuttings during season 2014-2015

| Character | Fresh weight /stems /plant (gm) | Dry weight/stems /plant (gm) | Moisture content /stems (%) |
|-------------|------------------------------------|---------------------------------|--------------------------------|
| Genotype 1 | 6.2 ^b | 1.68 ^b | 74.00 ^b |
| Genotype 2 | 7.78 ^b | 0.89 ^b | 79.65 ^b |
| Genotype 3 | 6.5 ^b | 1.3 ^b | 84.43 ^b |
| Genotype 4 | 7.97 ^b | 1.6 ^b | 79.92 ^b |
| Genotype 5 | 4.40 ^b | 0.87 ^b | 80.42 ^b |
| Genotype 6 | 6.12 ^a | 1.74 ^a | 81.67 ^a |
| Genotype 7 | 5.70 ^b | 1.38 ^b | 76.01 ^b |
| Genotype 8 | 4.57 ^b | 0.97 ^b | 78.93 ^b |
| Genotype 9 | 4.80 ^b | 0.97 ^b | 79.59 ^b |
| Genotype 10 | 3.18 ^b | 0.57 ^b | 81.98 ^b |
| Genotype 11 | 6.90 ^b | 1.16 ^b | 83.02 ^b |
| Genotype 12 | 6.65 ^b | 1.25 ^b | 78.96 ^b |
| Genotype 13 | 4.01 ^b | 0.88 ^b | 79.08 ^b |
| Genotype 14 | 4.52 ^b | 0.91 ^b | 79.73 ^b |
| Genotype 15 | 5.68 ^b | 1.36 ^b | 75.60 ^b |
| Genotype 16 | 8.42 ^b | 1.64 ^b | 80.71 ^b |
| Genotype 17 | 4.92 ^b | 1.01 ^b | 79.37 ^b |
| Genotype 18 | 7.32 ^b | 1.26 ^b | 82.85 ^b |
| Genotype 19 | 9.47 ^b | 2.15 ^b | 77.50 ^b |
| LSD=0.05 | 5.43 | 1.26 | 4.29 |

The results indicate the variation tended to appear similar in the same character. Some of the characters were more stable, namely the paired leaf numbers, number of nodes before transplanting, transplanting and plant height, number of nodes, length of leaf at harvest time. Some of the character was not stable, namely the number of branch and the yield of dry, fresh culm, leaf. The result of the analysis for character correlation was shown that some characters, closely related to yield, first of all were dry leaf weight, fresh leaf weight, fresh culm weight, dry culm weight per plant, then, culm width, number of branch, and the third, plant height, number of node, paired leaf number before transplanting. The results agree with Oliveira *et al.*, (2004) who examined some morphological features of strains of *Stevia rebaudiana*. The current research was in the line with Brandle and Rosa (1992) who studied the genetic variability in locally available stevia germplasm to allow selection of lines with agronomic and chemical properties suitable to local production conditions. They found that yield, leaf: stem ratio and stevioside concentration (62.1, 78.8 and 76.6, respectively) they were highly heritable. Yield levels were comparable to other stevia producing areas. Stevioside concentration was higher, probably as a result of production under long days. These initial results also showed that clearly suggested that genetic improvement of stevia is

possible. These high heritabilities enable selection and breeding programs, aimed at higher yield, to achieve substantial gains.

Extraction and estimation of Stevia sweeteners

Stevia sweeteners were analyzed and determined employing the high performance liquid chromatography HPLC. Data in Table 4 and Figures 1-2 showed the analysis of Stevia sweeteners (Stevioside (%) and rebaudioside-A). The results clearly indicated there were high significant variations between the nineteen genotypes under the study. The results showed that the highest concentration of rebaudioside-A was (28.2%) in genotype (6), and decreased to be (0.08% and 0.09%) in genotypes (12 and 13), respectively, as the lowest two genotypes in contrast in stevioside percentage the results showed that the highest percentage of stevioside was (99.5%) in genotype (12) followed by genotype (10) by (99.2%), and decreased to be (42.3% and 34.4% in genotypes (6 and 11) respectively. From the previous data, we can conclude that the different in rebaudioside A due to the different in the genetic background because these 19 genotypes were grown and polinated randomly for many different seasons. The data detected negatively correlation between stevioside and rebaudioside-A.

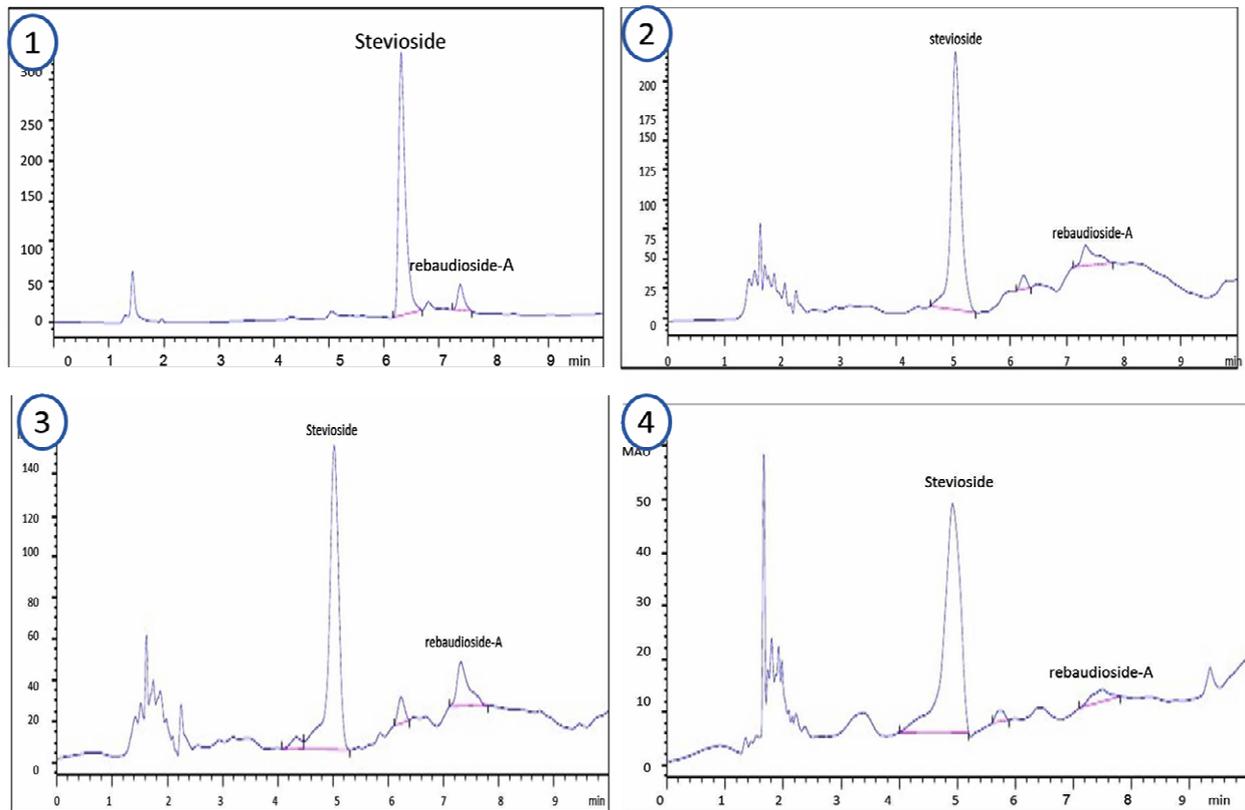


Figure 1. HPLC Chromatogram of stevioside and rebaudioside-A in relation to total sweeteners: (1) standard solution, (2) genotype_3, (3) genotype_4, and (4) genotype_5

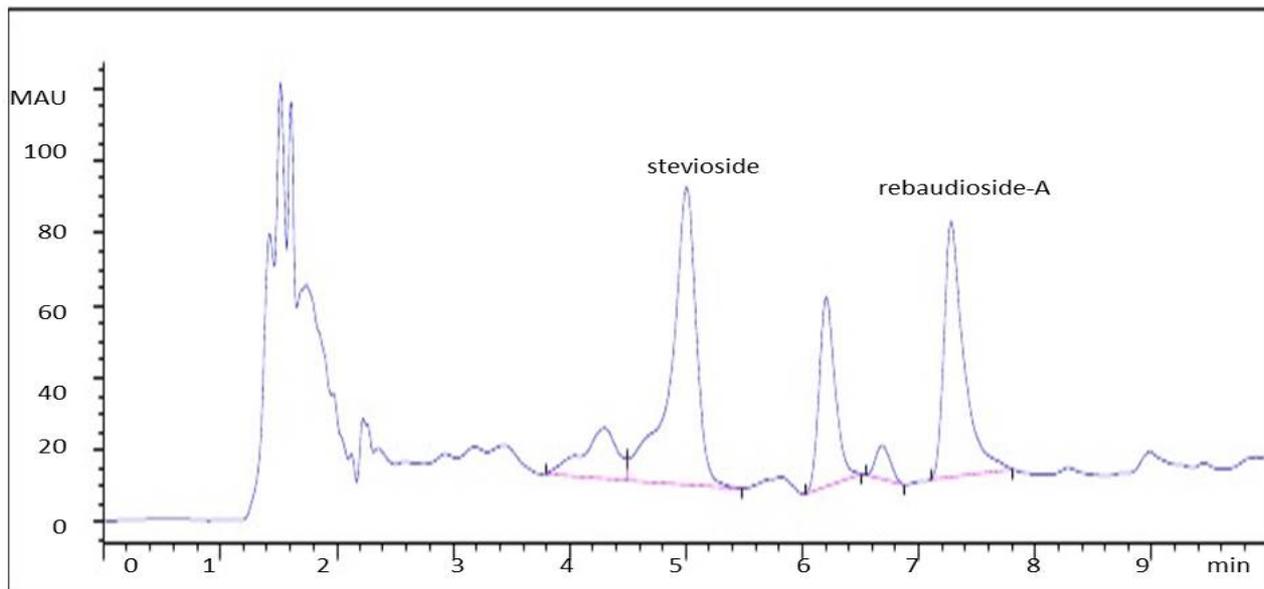


Figure 2. HPLC Chromatogram of stevioside and rebaudioside for genotype_6 in relation to total sweeteners

Table 4. Analysis of stevioside and Rebaudioside-A from *Stevia rebaudiana* fresh leaves using HPLC in relation to total sweeteners

| Genotype; | Stevioside (%) | Rebaudioside-A (%) |
|-------------|----------------|--------------------|
| Genotype_1 | 93.7 | 1.08 |
| Genotype_2 | 91.8 | 4.30 |
| Genotype_3 | 87.4 | 9.57 |
| Genotype_4 | 78.5 | 13.7 |
| Genotype_5 | 92.4 | 5.19 |
| Genotype_6 | 42.3 | 28.2 |
| Genotype_7 | 92.2 | - |
| Genotype_8 | 93.8 | - |
| Genotype_9 | 98.2 | - |
| Genotype_10 | 99.2 | - |
| Genotype_11 | 34.4 | 0.32 |
| Genotype_12 | 99.5 | 0.08 |
| Genotype_13 | 97.4 | 0.09 |
| Genotype_14 | 81.8 | 0.11 |
| Genotype_15 | 98.4 | 0.15 |
| Genotype_16 | 97.5 | 0.13 |
| Genotype_17 | 98.1 | 0.53 |
| Genotype_18 | 83.2 | - |
| Genotype_19 | 87.7 | 0.57 |

These correlations can be partially explained by the biosynthetic relationships between the individual glycosides because stevioside is the substrate for the synthesis of rebaudioside A. the plants which have high concentration in rebaudioside A will probably be low in stevioside.

These results are in agreement with Shibata *et al.*, (1995) who observed a wide range of variation in the four main glycosides and found that dulcoside A and stevioside, and rebaudioside A and C, and it were positively correlated with each other. Stevioside and rebaudioside A, and dulcoside and rebaudioside C, were negatively correlated with each other. These correlations can be partially explained by the biosynthetic relationships between the individual glycosides because stevioside is the substrate for the synthesis of rebaudioside A, plants high in rebaudioside A will probably be low in stevioside. The current results is agree with Huang *et al.*, (1995) who investigated the seedling population and clone variation from the difference of sweet component and content in *Stevia rebaudiana* Bertoni. The results showed: (1) there are 7.3% high yielding plants in seedling population, in which the proportion of R-A type with content of R-A over St content is 10.96%, and their R-A content varies from 3.3 to 12.0%. (2) Among the selected improved clone J-2, there are high significant difference in leaf size and steviosides content. The R-A content varies from 4.5 to 12.2%. (3) The individuals with R-A content of 7.04 apprx 12.03% from seedling population (original content 3.86%) and individuals with R-A

content of 10.15 apprx 12.15% from improved individual clone (original content 9.10%) were selected out.

RAPD-PCR ANALYSIS

Plant molecular geneticists are currently used RAPD markers routinely to identify genetic variations (Keil and Griffin, 1994; Lashermes *et al.* 1996; Irwin *et al.*, 1998 and Sun *et al.* 1998). RAPD markers have been also used successfully in various taxonomic and Phylogenetic studies. In addition, it locates regions of the genome linked to agronomically important genes (Reiter *et al.*, 1992; Martin *et al.*, 1991; Michelmore *et al.*, 1991; Pillay and Kenny 1996). Furthermore, it facilitates introgression of desirable genes into commercial accessions (Lavi *et al.*, 1994). In the present study, the genetic variability and relationships of different genotypes of *Stevia* (*Stevia rebaudiana*) have been studied based on RAPD analysis. Initial screening of large number of RAPD primers with nineteen genotypes of *stevia* (*Stevia rebaudiana*) resulted in eight RAPD primers that produced in formative and polymorphic products resolvable by agarose gel electrophoresis.

The results of primer OPN-04 are illustrated in Table 5. The total fragments were 61 amplification products at the fragment lengths ranged between 237 to 3000 bp. One of 61 fragments was monomorphic (300 bp) and 15 fragments were polymorphic i.e. (3000, 2841, 1825, 1707, 1413, 937, 895, 877, 822, 794, 790,

700, 529, 500 and 469 bp) and 45 fragments were unique (2684, 2377, 2230, 2088, 1598, 1500, 1338, 1273, 1220, 1128, 1073, 1065, 1040, 1019, 1000, 986, 982, 966, 950, 923, 900, 898, 886, 845, 842, 831, 810, 807, 790, 767, 737, 734, 718, 674, 656, 634, 632, 615, 581, 472, 438, 394, 378, 268 and 237 bp). The percentage of the polymorphism was 98.36%. Results showed that the genotypes number 2, 6, 7, 11, 12, 14, 15, 16 and 9 gave the same number of fragments (5, 6) respectively, While the genotypes 1, 8, 10, 17 and 18 detected the highest number of fragments (7 fragments). In the same time genotypes 5, 13 and 19 recorded the lowest number of fragments (4 fragments). On the other hand, the genotypes 3 doesn't amplified any fragments and he missing during loading procedure (Table 5)

The total fragments were 63 amplification products at the fragment lengths ranged between 127 to 2710 bp for OPD-05 primer. One monomorphic fragment was detected (1000 bp), 21 fragments were polymorphic and 41 fragments were unique. The percentage of the polymorphism was 98.4%. Results in Table 6 showed that genotypes 13 recorded (2 fragments, 3 (five fragments), while all the fowling genotypes (1, 18), (16, 17), (9, 15), (4, 10, 11) and (2, 6, 7, 8) detected the same number of fragments (1, 18), (16, 17), (9, 15), (4, 10, 11) and (2, 6, 7, 8), respectively. On the other hand genotype 3 detected 5 fragments. The highest numbers of DNA fragments were recorded to genotypes 14, 19 by 9 fragments. While, genotype 13 amplified the lowest DNA fragments number (2 fragments) and finally genotype 12 doesn't amplified any fragment.

The results of OPC-05 primer are illustrated in Table 7. The total fragments were 73 amplification products at the fragment lengths ranged between 100 to 2528 bp. Four fragments were monomorphic (1000, 700, 500 and 300 bp) and 18 fragments were polymorphic. The percentage of the polymorphism was 94.5%. Results in Table 7 showed that the highest number of DNA fragments was 13 fragments recorded to the genotypes 18. Genotypes 17 and 19 recorded 11 and 12 fragments, in respect. Genotypes (5, 8, 9), (2, 4, 15), (6, 7, 10, 13) and (14, 11, 16) detected the same number of fragments (7, 8, 9 and 10), respectively. On the other hand, the genotype 3, 12 amplified the lowest DNA fragments number (6 fragments). The total fragments by using primer OPB-07 were 28 amplification products at the fragment lengths ranged between 300 to 2429 bp. Two of 28 fragments were monomorphic (1435 and 801 bp) and seven fragments were polymorphic. 19 of 28 fragments were unique and the percentage of the polymorphism was 92.85%. (Table 8).

Results showed that genotypes 2, 6, 12, 15 and 4, 8, 9, 13, 16 detected the same number of fragments (3 and 4 fragments, respectively), while the genotypes 3, 7, 10, 11, 18 and 19 showed the highest number of DNA fragments (5 fragments). On the other hand, the genotype 14 amplified the lowest DNA fragments number (2 fragments). Genotypes 5, 17 don't gave any fragments.

The results of primer OPN-10 are illustrated in Table 9. The total fragments were 22 amplification products at the fragment lengths ranged between 628 to 2738bp. One fragment of 22 was monomorphic (1015 bp), six of 22 fragments were polymorphic (2738, 995, 898, 890, 883 and 628 bp) and 15 fragments were Unique (2483, 1781, 1679, 1585, 983, 939, 909, 900, 876, 830, 787, 756, 688, 680 and 648 bp). The percentage of the polymorphism was 95.45%. Results showed that genotypes (2, 5, 7, 9, 11, 14, 17, 19) and genotypes (3, 4, 6, 10, 18) detected the same number of fragments (2 and 3 fragments), respectively, while, the genotypes 1, 15 and 16 showed the highest number of DNA fragments (4 fragments). On the other hand, the genotypes 8, 12 and 13 don't gave any fragments.

Concerning to primer OPN-10 found in Table 10, the total fragments were 17 amplification products at the fragment lengths ranged between 113 to 1405 bp. one of 17 fragments was monomorphic (500 bp), one of 17 fragments was polymorphic (665 bp) and 15 fragments were unique. The percentage of the polymorphism was 94%. With primer OPQ-12 the total fragments were 19 amplification products at the fragment lengths ranged between 176 bp. to 1018 bp. one of 19 fragments was monomorphic (714 bp), 4 of 19 fragments was polymorphic (775, 742, 728, and 176 bp) and 14 fragments were unique. The percentage of the polymorphism was 94.7 % (Table 11). The results of primer OPQ-14 are illustrated in Table 12. The total fragments were 9 amplification products at the fragment lengths ranged between 190 bp. to 2316 bp. One of 9 fragments was monomorphic (190 bp), 2 of 9 fragments were polymorphic (612 and 829 bp) and 6 fragments were unique (2316, 1406, 926, 641, 222 and 197 bp). The percentage of the polymorphism was 89%.

Data in Table 13 showed the primer name, total number of amplicons, monomorphic and polymorphic amplicons and the percentage of polymorphism among the nineteen genotypes. The mean percentage of polymorphism was 94.65 %.

Table 5. DNA polymorphism of nineteen genotypes of *Stevia rebaudiana* plant using randomly amplified polymorphic DNA with primer OPN-04

| Genotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---------------|----|----|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Monomorphic | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Polymorphic | 2 | 0 | 0 | 2 | 0 | 1 | 2 | 3 | 3 | 2 | 2 | 3 | 2 | 1 | 1 | 1 | 1 | 2 | 2 |
| Unique | 4 | 4 | 0 | 3 | 3 | 3 | 2 | 4 | 2 | 4 | 2 | 1 | 1 | 3 | 3 | 3 | 4 | 4 | 3 |
| Total bands | 7 | 5 | 0 | 6 | 4 | 5 | 5 | 7 | 6 | 7 | 5 | 5 | 4 | 5 | 5 | 5 | 7 | 7 | 4 |
| Polymorphism% | 85 | 80 | 0 | 83 | 75 | 80 | 80 | 85 | 83 | 85 | 80 | 80 | 75 | 80 | 80 | 80 | 85 | 85 | 75 |

Table 6. DNA polymorphism of nineteen genotypes of *Stevia rebaudiana* using randomly amplified polymorphic DNA with primer OPD-05

| Genotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---------------|------|------|----|------|----|------|------|------|----|------|------|----|----|----|----|----|----|------|------|
| Monomorphic | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Polymorphic | 1 | 3 | 2 | 5 | 3 | 6 | 4 | 7 | 4 | 3 | 3 | 0 | 1 | 3 | 1 | 2 | 1 | 1 | 1 |
| Unique | 1 | 4 | 2 | 1 | 2 | 1 | 3 | 0 | 1 | 3 | 3 | 0 | 0 | 5 | 4 | 2 | 3 | 3 | 5 |
| Total bands | 3 | 8 | 5 | 7 | 6 | 8 | 8 | 8 | 6 | 7 | 7 | 0 | 2 | 9 | 6 | 5 | 5 | 3 | 7 |
| Polymorphism% | 66.6 | 87.5 | 80 | 85.7 | 83 | 87.5 | 87.5 | 87.5 | 83 | 85.7 | 85.7 | 0 | 50 | 89 | 83 | 80 | 80 | 66.6 | 85.7 |

Table 7. DNA polymorphism of the nineteen Genotypes of *Stevia rebaudiana* plant using randomly amplified polymorphic DNA with primer OPC-05

| Genotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---------------|----|----|----|----|------|------|------|------|------|------|----|------|------|------|----|----|------|----|------|
| Monomorphic | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Polymorphic | 2 | 1 | 1 | 1 | 3 | 2 | 2 | 2 | 0 | 3 | 3 | 2 | 2 | 5 | 1 | 1 | 2 | 3 | 5 |
| Unique | 4 | 1 | 1 | 3 | 0 | 3 | 3 | 1 | 3 | 2 | 3 | 3 | 0 | 6 | 3 | 3 | 4 | 4 | 6 |
| Total bands | 10 | 6 | 6 | 8 | 7 | 9 | 9 | 7 | 7 | 9 | 10 | 9 | 9 | 11 | 8 | 10 | 11 | 13 | 12 |
| Polymorphism% | 60 | 33 | 33 | 50 | 42.8 | 55.5 | 55.5 | 42.8 | 42.8 | 55.5 | 60 | 55.5 | 55.5 | 63.6 | 50 | 60 | 63.6 | 69 | 66.6 |

The genetic polymorphism between the nineteen genotypes ranged from 89% by using OPQ-14 to 98.4% with OPN-04 and OPD-05, in respect (Table 13). That mean there are high genetic diversity between the current genotypes and it could be used in the breeding program to obtaining new genetic resources from *Stevia rebaudiana* plants.

To study the genetic similarities and phylogenetic relationships among the nineteen tested genotypes of *Stevia rebaudiana* RAPD-PCR analysis were used. The obtained data were subjected to cluster analysis with dice equation by using NTYSIS (ver 2.1) computer program to calculate proximity matrix and design dendrogram. In the present study the genetic relationships among the nineteen genotypes were determined by Dice coefficient. The matrix of similarity index ranged from 0.78 recorded between genotype 13 and 14 to 0.91 recorded between genotype 7 and 8 with an average of 0.80. The dendrogram illustrating the distance among the examined genotype is presented in Figure 3. The dendrogram in this case revealed two main groups (A & B) by 84% similarity. The first group A consists of two clusters (genotype 9 and 3) by 84 % similarity. Cluster B divided to two sub clusters (A' and B') by 82.5% similarity. Sub cluster (A') divided to two three clusters and includes (Genotypes 19, 18, 16, 13) by 83% similarity. Sub cluster (B') divided to two sub cluster (A'' & B''). Sub cluster A'' includes two sub clusters AND genotypes 6, 7, 8, 10, 11, 14 and 15, while, sub cluster B'' include both genotypes 1, 2, 4, 5, 12 and 17.

The current study was in the line with Muktaduzzaman and Rahman (2009) who worked on 9 randomly selected regenerated plants and subjected to RAPD analysis. Their results indicated that the estimation of genetic similarity coefficient based on RAPD band-sharing data indicated that some regenerated plants were 100% similar to the mother the mother plants and some were 71.57 or 14% similar may be due to in vitro condition. Also, our results are agree with Radhika *et al.*, (2012) who worked on molecular genetic fingerprints of medicinal species using

Randomly Amplified Polymorphic DNA (RAPD) marker to elucidate the genetic diversity among the 18 species of *stevia rebaudiana* plant. The results indicated that of which 232 fragments were polymorphic with 96.84% of polymorphism. Some of the RAPD markers were useful for species discrimination and identification. Most of the RAPD markers studied showed different level of genetic polymorphism. Amplified fragment sizes ranged from 300 to 5000 bp. PairwiseNei and Li's similarity coefficient value ranged from 0.00-0.72 for 18 species of medicinal plants. The results in the line with Chester *et al.*, (2013) who studied the genetic and metabolic variability in *S. rebaudiana* among accessions of different geographical regions of India using random amplified polymorphic DNA (RAPD) markers. They found ten out of 20 primers screened were most informative; amplification products of the genotypes yielded a total of 87 scorable bands (67 polymorphic), whereas genetic similarity (GS) coefficient (0.01-0.08) and polymorphism (67.24-92.40%) showed huge variability. The current results in the line with Paramanik and Chikkaswamy (2014) who assayed the genetic relationships between eighteen medicinal plants with RAPD markers. They showed that over 77.23 reproducible bands were generated by RAPD primers, out of which, 77.23 polymorphic bands were identified, conferring 97.6% polymorphism. All the primers produced typical banding in each of the medicinal plants, suggesting the applicability of this test in medicinal plant identification. Results of the current work is agreed with Chikkaswamy (2015) who investigated molecular genetic fingerprints of medicinal species using RAPD marker to elucidate the genetic diversity among the 18 species. Three decamer-primers generated 250 RAPD fragments, of which 232 fragments were polymorphic with 96.84% of polymorphism. Some of the RAPD markers were useful for species discrimination and identification. They maintained that most of the RAPD markers studied showed different level of genetic polymorphism. Amplified fragment sizes ranged from 300 to 5000 bp.

Table 12. DNA polymorphism of Nineteen genotypes of *Stevia rebaudiana* plant using randomly amplified polymorphic DNA with primer OPQ-14

| Genotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---------------|------|---|---|---|---|----|----|---|---|------|----|----|----|----|----|----|----|----|----|
| Monomorphic | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| Polymorphic | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unique | 2 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Total bands | 3 | 0 | 1 | 0 | 0 | 2 | 2 | 1 | 1 | 3 | 4 | 0 | 0 | 2 | 0 | 1 | 0 | 1 | 0 |
| Polymorphism% | 66.6 | 0 | 0 | 0 | 0 | 50 | 50 | 0 | 0 | 66.6 | 75 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 0 |

Table 13. Polymorphism data as detected by RAPD markers, total number of amplicons, monomorphic and polymorphic amplicons and the percentage of polymorphism among the nineteen genotypes

| polymorphism % | unique | polymorphic | Monomorphi c | Total amplicons | Primer code |
|----------------|--------|-------------|-----------------|-----------------|----------------|
| 98.4 | 45 | 15 | 1 | 61 | OPN-04 |
| 98.4 | 41 | 21 | 1 | 63 | OPD-05 |
| 94.5 | 51 | 18 | 4 | 73 | OPC-05 |
| 92.8 | 19 | 7 | 2 | 28 | OPB-07 |
| 95.5 | 15 | 6 | 1 | 22 | OPN-10 |
| 94.0 | 15 | 1 | 1 | 17 | OPG-12 |
| 94.7 | 14 | 4 | 1 | 19 | OPQ-12 |
| 89.0 | 6 | 2 | 1 | 9 | OPQ-14 |
| | 206 | 74 | 12 | 292 | Total |
| 94.65 | | | | | Polymorphism % |

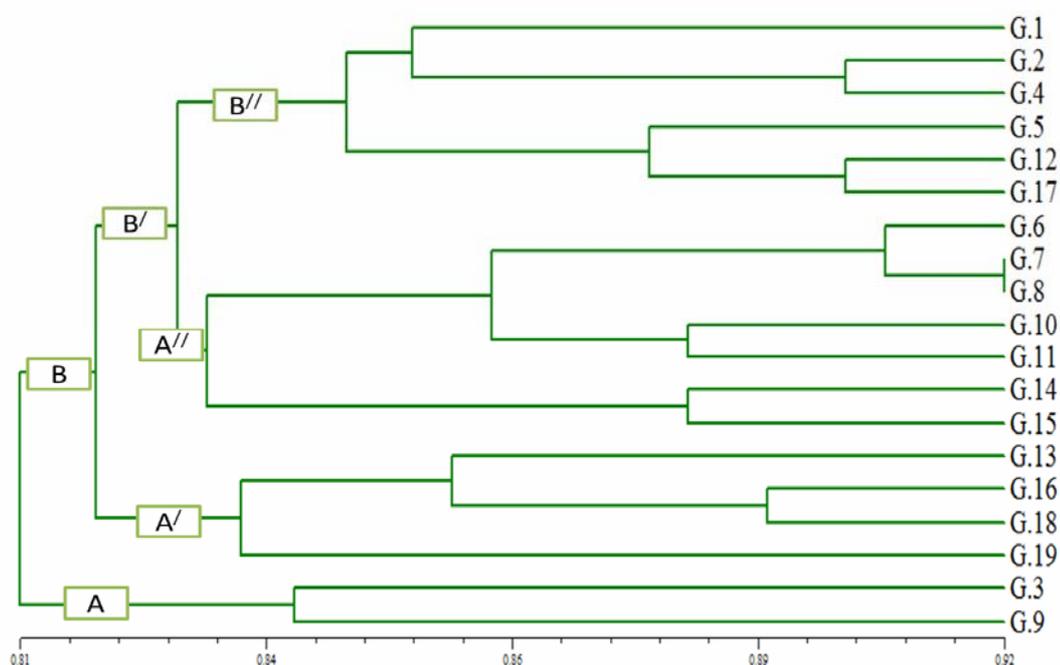


Figure 3. Dendrogram of similarity of different nineteen Stevia genotypes based on 8 RAPD-RCR primers

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